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ORIGINAL ARTICLE

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PankoMab: a potent new generation anti-tumour MUC1 antibody

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Abstract Recently, we described a new carbohydrate-induced conformational tumour-epitope on mucin-1 (MUC1) with the potential for improvement of immunotherapies [29, 30]. PankoMab is a novel antibody, which binds specifically to this epitope and was designed to show the highest glycosylation dependency and the strongest additive binding effect when compared to other MUC1 antibodies. This enables PankoMab to differentiate between tumour MUC1 and non-tumour MUC1 epitopes. It has a high-affinity towards tumour cells (e.g. K_D [M] of 0.9 and 3×10^{-9} towards NM-D4 and ZR75-1, respectively) and detects a very large number of binding sites (e.g. 1.0 and 2.4×10^6 for NM-D4 and ZR75-1, respectively). PankoMab is rapidly internalised, and after toxin coupling is able to induce very effectively toxin-mediated antigen-specific tumour cell killing. PankoMab reveals a potent tumour-specific antibody-dependent cell cytotoxicity (ADCC). PankoMab is, therefore, distinguished by a combination of advantages compared to other MUC1 antibodies in clinical development, including higher tumour specificity, higher affinity, a higher number of binding sites, largely reduced binding to shed MUC1 from colon and pancreatic carcinoma patients, no binding to mononucleated cells from peripheral blood (except ~7% of activated T cells), stronger ADCC activity and rapid internalisation as required for toxin-mediated cell killing. This renders it a superior antibody for in vivo diagnostics and various immunotherapeutic approaches.

Keywords PankoMab · MUC1 · Antibody · Immunotherapy

Abbreviations ADCC: Antibody-dependent cell cytotoxicity · MUC1: Mucin-1 · PBMC: Peripheral blood mononuclear cells

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Introduction

Mucin-1 (MUC1) is an established tumour marker expressed on a variety of epithelial tumours. Besides its use as a serum tumour marker, especially in breast cancer patients [23, 40], it has attracted increasing attention as a potential target of tumour immunotherapies [19, 45].

Mucin-1 is a large highly *O*-glycosylated transmembrane glycoprotein. The extracellular portion consists of a variable number of 20–120 tandem repeats (TR). Each TR consists of 20 amino acids with five potential *O*-glycosylation sites (for review see [4, 26]).

Three major features render MUC1 a tumour target for antibody therapies. (1) In epithelial tumours, MUC1 becomes strongly overexpressed, (2) the strictly apical expression, which occurs on most normal epithelia, is lost in the tumour, where MUC1 is expressed apolarly over the whole cell surface, rendering it accessible by systemically administered antibodies, (3) an aberrant *O*-glycosylation in the tumour exposes new peptide epitopes on the MUC1 protein backbone and new carbohydrate tumour antigens such as the Thomsen–Friedenreich (TF) antigen [6, 9–13, 21, 28, 34, 45].

While these facts are in favour of MUC1 as a target for antibody therapies, there are several more problematic aspects, which arose more recently. It was found that MUC1 is not only expressed on certain epithelial tissues, but also on several cells of the haematopoietic

system. In addition, MUC1 shed into the serum of cancer patients in considerable amounts can be bound by certain therapeutic antibodies, reducing their therapeutic effect [1, 7, 8, 14–16, 18, 46, 47].

In 1996, a panel of 56 monoclonal anti-MUC1 antibodies were compared at the ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1 [39]. These results as well as further studies [13] clearly showed that all antibodies had different fine-specificities. Even those antibodies against the immunodominant DTR region, recognising nominally the same peptide region, proved to express not only different binding patterns in histology, flow cytometry and serology, but also in biochemical fine mapping of the epitope, showing that each anti-MUC1 antibody is different [28].

More recently, Karsten et al. [29, 30] described a novel carbohydrate-induced conformational tumour epitope on MUC1 (TA-MUC1) of the structure ...PDT*RP..., where T* is *O*-glycosylated with GalNAcα1- or a similar short, non-sialylated glycan such as Galβ1-3GalNAcα1- (core-1). The glycans present at this site are themselves tumour-specific carbohydrate structures [21, 34], and also play a critical role in the induction of the specific conformation of the epitope. From the 22 antibodies recognising the immunodominant DTR motif, all antibodies (11) generated from tumour-derived MUC1 showed enhanced binding to peptides with glycosylated DTR (glycosylation effect). In contrast, those antibodies generated with non-tumour MUC1 did not differentiate between glycosylated and non-glycosylated peptides. Interestingly, the knob-like structure shown by repetitive non-glycosylated MUC1 TR peptides [20] is not identical to the carbohydrate-induced conformational epitope [29, 30].

These studies also showed that antibodies against MUC1 currently in clinical development (HMFG-1 and C595), which were made by immunisation with MUC1 from non-tumour sources, express a specificity different from the novel tumour epitope on MUC1, and are, therefore, not sufficiently discriminating between normal and tumour MUC1.

Therefore, our aim was to generate and characterise a MUC1 antibody for anti-tumour immunotherapies that maximally discriminates between the novel conformational MUC1 tumour epitope and the non-glycosylated MUC1 epitope, as shown by the highest glycosylation effect and the highest additive length effect. Here, we demonstrate that PankoMab meets these criteria and shows a combination of favourable features that makes it superior for further development for in vivo diagnostics and tumour therapy.

Materials and methods

Cell culture

The human breast cancer cell line MCF-7 (ATCC No HTB 22) was cultured in DMEM supplemented with 10% foetal calf serum (FCS) and 2 mM glutamine. NM-D4 (a human leukaemia cell line, which was glycoengineered in order to express the TF antigen and the glycosylated PDTRP tumour epitope TA-MUC1, manuscript in preparation) and the human breast carcinoma cell lines T-47D (ECACC No 85102201), ZR-75-1 (ATCC No CRL 1500) and MT-3 (DSMZ No ACC 403) were cultured in RPMI 1640 supplemented with 10% FCS and 2 mM freshly added glutamine. Hybridoma cells were routinely grown in RPMI 1640 supplemented with 5% FCS and 2 mM glutamine. All cells were grown at 37°C in a humidified atmosphere of 6% CO_2. Media and supplements were purchased from Biochrom, Germany.

Generation of PankoMab

PankoMab was generated by a procedure consisting of two critical steps: (1) immunising Balbc mice with tumour MUC1 from a desialylated human breast cancer source, to generate hybridomas according to a method described by Karsten et al. [27] and selection of hybridomas with high binding to the glycopeptide APPAHGVT-SAPDT[GalNAcα]RPAPGSTAPPAHGVTSA and low binding to its unglycosylated counterpart by ELISA (sensitive to the glycosylation effect; i.e. specific for the tumour MUC1 epitope); (2) further improvement of the specificity by using multiple rounds of extensive reclon-ing, and selection for the highest glycosylation effect and the highest ratio of binding to A[HGVTSAPDT(GalNAcα)RPAPGSTAPPA]$_4$ versus AHGVTSAPDT(GalNAcα)RPAPGSTAPPA (additive length effect). The most suited clone was called PankoMab. The isotype of PankoMab was determined as IgG1, κ by a commercial kit (BD Bioscience Pharmingen, Germany).

Purification of PankoMab

Hybridoma cells were maintained in Hybridoma Express medium (PAA, Germany) supplemented with 2 mM glutamine. Purification was performed by protein A affinity chromatography using an elution buffer at pH 3.5. The purified antibody was quantified by UV measurement and analysed by ELISA and SDS-polyacrylamide electrophoresis.

Other MUC1 antibodies

HMFG-1 (mIgG1) was purchased from Immunotech, France, VU-3C6 (mIgG1) from Biogenesis, UK, and 115D8 (mIgG2b) and DF3 (mIgG1) were from DPC Biermann, Germany.

Serum samples

Blood samples from patients with adenocarcinoma of the pancreas were collected at the Department of

Surgery, University of Heidelberg, Germany. Blood samples from colon carcinoma patients were collected at the Robert-Rössle-Klinik, Berlin-Buch, Germany. Samples were obtained prior to medical intervention and the sera were frozen immediately until further analysis.

Peptides and glycopeptides

Several synthetic peptides/glycopeptides, all based on MUC1 TR sequences, were employed for different sets of experiments.

1. A Tn (GalNAcα)-glycosylated 30-mer with the sequence APPAHGVTSAPDT[GalNAcα]RPAPGS TAPPAHGVTSA and its unglycosylated counterpart were synthesised by Biosyntan (Berlin, Germany).
2. A series of Tn-glycosylated MUC1 TR peptides of different length with the sequences A[HGVTSA PDT(GalNAcα)RPAPGSTAPPA]$_n$, with $n = 1$–5 (TR1a–TR5a), were synthesised by H. Paulsen and N. Serttas (Institute of Organic Chemistry, University of Hamburg, Germany) as described in Karsten et al. [29].

Enzyme immunoassays

Enzyme immunoassays (ELISAs) were performed as described in Karsten et al. [29]. In all ELISA experiments, coating was done on a weight per mL basis, in order to approximate equimolar concentrations of TRs. Blank values were subtracted, and means were calculated. Each experimental type was performed multiple times and with different concentrations of antibodies and antigens. In some cases, carbohydrate-selective mild periodate oxidation of the coated antigens as described in Woodward et al. [48] (10 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, for 1 h at 25°C, followed by reduction of aldehydes by 50 mM $NaBH_4$ in PBS) was performed.

Tumour cell binding experiments and affinity determination

Purified PankoMab was chelated with p-SCN-benzyl -DTPA and radiolabelled with ^{111}In according to Nikula et al. [35]. Labelling was done with carrier-free ^{111}In to specific activities of 7–37 MBq/mg. Binding assays were performed in duplicate with 1×10^6 cells of ZR-75-1, MCF-7, T-47D and NM-D4 in 1.5 mL tubes and increasing serial dilutions of labelled antibody (320–5 ng) in a total volume of 200 µL PBS supplemented with 1% bovine serum albumin (BSA) to avoid non-specific binding. The cells were incubated for 1 h at 4–8°C, separated from unbound antibody by centrifugation at 4,000×g for 3 min and washed 2 times with 200 µL PBS/1% BSA. Cell-bound radioactivity was quantified in a gamma-counter. Comparability of immunoreactivity of labelled and unmodified PankoMab was confirmed by competition of ^{111}In-PankoMab with PankoMab using the same experimental protocol. 5 ng ^{111}In-PankoMab was added to increasing amounts of PankoMab and incubated with the cells. PankoMab binding affinity and capacity were calculated for each individual cell line by Scatchard plot analysis. HMFG-1 was chelated and labelled as described for PankoMab and compared in parallel experiments.

PBMC isolation from blood donors

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by density centrifugation with Ficoll-Hypaque (Biochrom, Germany). The cells were washed 3 times with RPMI 1640 supplemented with 5% FCS and cryopreserved in separate batches of 5×10^7 cells. The PBMC were thawed and used directly or kept overnight in RPMI 1640 supplemented with 10% FCS (RPMI/FCS) before use in flow cytometry or as effector cells in the antibody-dependent cell cytotoxicity (ADCC) assay. Mitogen stimulation was performed by 3–8 days incubation of isolated PBMC in RPMI/FCS containing 1 µg/mL phytohemagglutinin (Sigma, Germany) and 60 U/mL human interleukin-2 (PeproTech, UK). Stimulation was controlled by detection of the CD25 activation marker in flow cytometry.

Flow cytometry

The isolated PBMC and ZR-75-1 cells were suspended in RPMI/FCS and incubated with non-conjugated monoclonal antibodies (10 µg/mL) on ice for 1 h. The cells were then washed twice with PBS and incubated on ice for 30 min in the dark with Cy3-conjugated goat anti-mouse Ig (Dianova, Germany) and/or with directly conjugated antibodies (BD, Belgium) to CD3 (T cells), CD14 (monocytes), CD19 (B cells) or CD25 (activated T cells) to discriminate between the different cell populations. After washing the cells twice with PBS, flow cytometric analysis was performed using a Coulter Epics XL flow cytometer (Beckman Coulter, Germany).

Detection of MUC1 in serum samples

The serum MUC1 levels of 22 patients with pancreatic carcinoma and 24 with colon carcinoma were detected using a sandwich immunoassay. Briefly, 96-well plates (MaxiSorb, Nunc, Germany) were coated with 50 µL per well of 1 µg/mL anti-MUC1 antibody (PankoMab, 115D8 or HMFG-1) in PBS overnight at 4°C, blocked with PBS/0.05% Tween-20 containing 5% BSA, washed and incubated for 1.5 h at room temperature with 50 µL per well of different serum samples. Biotinylated antibodies PankoMab, DF3 and HMFG-1 were added

(0.5 μg/mL) and incubated for 1 h at room temperature. After washing and incubation with peroxidase-labelled streptavidin (Dianova, Germany), the wells were developed with TMB (tebu-bio, Germany) and measured at 450 nm. All probes were compared to a standard of 23 U/ml MUC1 as a cut-off level for healthy controls, which was kindly provided by S. von Mensdorff-Pouilly, Vrije University Amsterdam, the Netherlands.

Internalisation of PankoMab by tumour cells

Internalisation was investigated for two tumour cell lines (T-47D, ZR-75-1) using the ^{111}In-labelled antibody. The tumour cells were precooled on ice for 1 h and loaded with antibody (typically 300 ng PankoMab per 5×10^5 cells). Unbound antibody was washed away by centrifugation. Aliquots of cells were incubated in triplicate at 4 or 37°C. The incubation was started by the addition of 50 μL cell suspension to 150 μL incubation buffer of the desired temperature and storage at this temperature. At various times, the supernatants were separated by centrifugation (2 min, 6,000×g) and aspiration (S1). Afterwards, 200 μL ice-cold strip-buffer (50 mM glycine, 150 mM NaCl, pH 2.5) was added per vial to remove the cell surface bound antibody. The vials were incubated for 10 min on ice, centrifuged again and washed with cold PBS/1% BSA (S2). Radioactivities of supernatants as well as cell pellets were quantified. The acid resistant radioactivity measured was calculated and corresponds to the amount of internalised antibody. Radioactivity in the supernatant S1 represents the unbound as well as shed antibody and that of supernatant S2 the stripped former cell surface bound amount of antibody at the individual times. Internalisation was calculated as percentage of total bound PankoMab. Cells that do not express MUC-1 served as the controls.

Toxin-mediated cell killing

Cell death mediated by internalisation of toxin-coupled PankoMab was analysed using the β-amanitin-conjugated PankoMab named ToxiMab. ToxiMab and β-amanitin were kindly provided by Professor H. Faulstich, Max-Planck-Institut für Medisinische Forschung, Heidelberg, Germany. Tumour cells (T-47D and ZR-75-1) were grown for 2 days (~ half-confluent) in a 96-well tissue culture plate (Nunc, Germany) and treated with medium as a control or either β-amanitin, PankoMab, ToxiMab or β-amanitin-conjugated mouse IgG as a control antibody in different concentrations at 37°C in a humidified atmosphere. Incubation was stopped either after 1, 4 or 24 h by removing the supernatant compietely and replacing it with fresh medium. Quantification of cell death was performed using the WST-1 proliferartion assay (Roche, Germany). Cell viability was calculated as percentage of untreated cells (mean of four replicates).

Antibody-dependent cell cytotoxicity

Cytotoxicity of PankoMab was analysed in europium (Eu^{3+})-release assay. The target cells (5×10^6) were incubated for 10 min at 4°C in 800 μL of europium solution (50 mM HEPES, pH 7.4, 93 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM diethylenetriamine-pentaacetic acid, 2 mM europium (3)-acetate), electroporated at 710 V, 1 pulse, 30 μs using a Multiporator from Eppendorf (Germany) and incubated for another 10 min on ice. The cells were washed in 50 mL and 4 times in 15 mL of RPMI 1640 medium supplemented with 5% FCS (RPMI/FCS). The target cells (5×10^3 in 100 μL RPMI/FCS per well) were seeded in triplicates in 96-well tissue culture plates (round bottom, Nunc, Germany) before the addition of 20 μL RPMI/FCS or PankoMab in RPMI/FCS, giving a final concentration of 0.2–5 μg/mL in a complete incubation volume of 200 μL. Then, 80 μL/well of PBMCs in RPMI/FCS in various effector-to-target ratios (E/T) or RPMI/FCS (to determine spontaneous lysis) were added. The plates were centrifuged for 1 min at 500×g and incubated for 4 h or overnight at 37°C in a humidified atmosphere of 6% CO_2. To determine maximal lysis, 100 μL target cell suspension was incubated with 100 μL 96% ethanol. The cells were centrifuged for 5 min at 500×g, and 20 μL culture supernatant was collected and added to 200 μL/well of enhancement solution (PerkinElmer Wallac, Germany) in 96-well tissue culture plates (Flat bottom, Nunc, Germany). After incubation for 15 min at room temperature, fluorescence was determined using a Victor^2 fluorometer (PerkinElmer Wallac, Germany). Specific cytotoxicity was calculated as percentage cytotoxicity = (experimental lysis–spontaneous lysis)×100/(maximal lysis–spontaneous lysis).

Results

Biochemical characterisation of the fine-specificity of PankoMab

Binding characteristics of the MUC1-specific antibody PankoMab were analysed using different glycosylated and non-glycosylated MUC1-derived tandem repeat peptides in ELISA studies (Fig. 1). PankoMab strongly binds to a short MUC1 peptide of 30 amino acids comprising 1.5 TRs when it is glycosylated with GalNAcα at the T of the PDTRP-sequence, but not if the same peptide is not glycosylated (Fig. 1a). No qualitative differences were seen using direct coating to the polystyrene surface or coating via biotin/streptavidin using corresponding biotinylated glycopeptides/peptides. PankoMab does not bind to short MUC1 peptides, which are glycosylated at other serines or threonines of the TR instead of PDTRP (data not shown). PankoMab does not bind to peptides consisting of 1–2 TRs with no PDTRP glycosylation, but shows

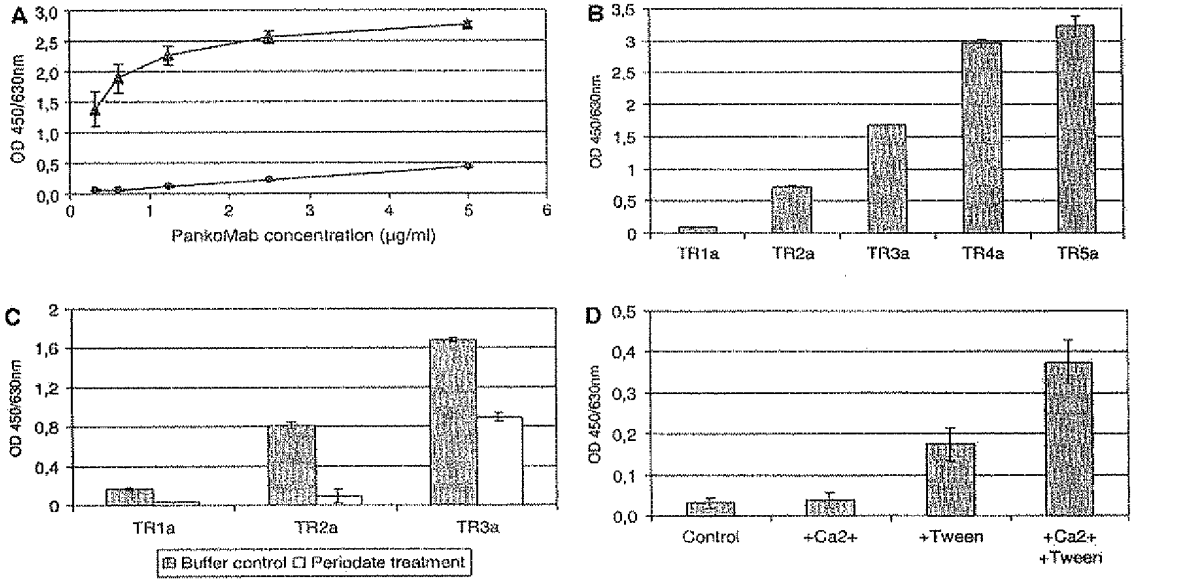

Fig. 1 Biochemical characterisation of the fine-specificity of PankoMab. **a** Glycosylation-dependent binding of PankoMab. PankoMab binds strongly to a 30-mer MUC1-derived peptide comprising 1.5 TRs with GalNAcα-glycosylation at the T of the PDTRP-sequence (*filled triangle*), but shows only non-specific binding to the non-glycosylated counterpart (*filled circle*). **b** PankoMab binding towards multiple PDTRP-glycosylated tandem repeat peptides TR1a-TR5a. **c** Reduction of PankoMab binding towards multiple PDTRP-glycosylated TR peptides TR1a–TR3a following periodate treatment. **d** PankoMab binding to the PDTRP-glycosylated 30-mer peptide (coated in this case from aqueous solution to dryness) after renaturation of the conformational epitope by incubation in PBS in the presence or absence of Ca^{2+} and/or Tween

weak binding towards multiple non-glycosylated TRs (data not shown). The strong binding of PankoMab towards the PDTRP-glycosylated TR (Fig. 1a) is further increased with the increasing length of glycopeptides (with multiple PDTRP-glycosylated TRs, Fig. 1b). We refer to this phenomenon as the additive effect of glycosylation and length. When comparing Fig. 1a, b, it should be mentioned that the glycopeptide TR1a is shorter than the glycopeptide used in Fig. 1a, and in fact too short to establish the tumour-specific conformation. Mild periodate oxidation in combination with borohydride treatment, which cleaves the sugar ring and abolishes its impact on epitope conformation, was used for further dissection of the glycosylation and length effects. As shown in Fig. 1c, glycosylation was solely responsible for the binding of PankoMab to the dimer (TR2a), whereas in the case of the trimer (TR3a), the length effect also came into action. The conformational nature of the PankoMab epitope was further confirmed by renaturation studies. A PDTRP-glycosylated MUC1 peptide (as in Fig. 1a) dried from an aqueous solution onto the ELISA plate overnight was renatured by incubation with PBS ± Ca^{2+} and/or Tween-20. While Ca^{2+} alone did not result in a substantial renaturation of the conformational epitope, Tween-20 alone did, but the best results were obtained with both Ca^{2+} and Tween-20 (Fig. 1d).

Determination of cell binding affinity

PankoMab binding to tumour cell lines was evaluated using radiolabelled antibody in cell binding studies. The immunoreactivity of PankoMab was not changed by radiolabelling as shown by ELISA and competition experiments. The dissociation constant and the antibody binding sites were estimated by Scatchard plot analysis. Four different tumour cell lines were analysed: the breast carcinoma cell lines ZR-75-1, MCF-7 and T-47D and the leukaemia-derived cell line NM-D4. The obtained data are summarised in Table 1. PankoMab binds with comparable and high affinities to all the cell lines investigated. The results show equilibrium constants in the lower nanomolar to subnanomolar range. The number of antibody binding sites was calculated in the range of 10^6 per cell. For comparison, the experiments were also performed with the antibody HMFG-1 under the same conditions. On the whole, the affinity of HMFG-1 and the average number of antibody molecules bound per cell were about one magnitude less when compared to PankoMab.

Flow cytometric analysis of human PBMC

PankoMab binding to MUC1 expressed on human PBMC was investigated using flow cytometry and

Table 1 Cell binding affinity and binding sites per cell of PankoMab compared to HMFG-1 on different tumour cell lines

Tumour cell line	Dissociation constant K_D [M]		Binding sites per cell		Number of individual experiments (N)	
	PankoMab	HMFG-1	PankoMab	HMFG-1	PankoMab	HMFG-1
ZR-75-1	3.03×10^{-9}	1.22×10^{-8}	2.40×10^{6}	2.10×10^{5}	12	2
MCF-7	3.84×10^{-9}	1.19×10^{-8}	0.44×10^{6}	0.35×10^{5}	7	2
T-47D	7.14×10^{-9}	1.92×10^{-8}	1.60×10^{6}	1.40×10^{5}	6	6
NM-D4	0.91×10^{-9}	0.37×10^{-8}	1.00×10^{6}	0.63×10^{5}	17	6

Data were estimated by Scatchard plot analysis

compared to HMFG-1. Binding to monocytes (CD14), B cells (CD19) and T cells (CD3) and to mitogen-activated T cells (CD3, CD25) was determined (Fig. 2). The granulocytes were not analysed, because MUC1 was described to be absent as determined by RT-PCR and antibody staining [7, 18]. PankoMab revealed no binding to the cells except to about 7% of mitogen-activated T cells if the background binding, which was measured with a murine isotype control, was subtracted. In contrast, HMFG-1 showed considerable binding to B cells, monocytes and even higher binding to activated T cells (Fig. 2a). The binding of PankoMab to mitogen-activated T cells was very weak in contrast to the very strong binding to MUC1 expressing tumour cells as shown with ZR-75-1 (Fig. 2b). The percentage of bound cells can vary between freshly prepared PBMC and those from cryopreserved samples, as well as among different donors. Other MUC1 antibodies revealed different binding patterns, e.g. VU-3C6 did not bind to T cells or activated T cells but showed strong binding to monocytes, which amounted to nearly 100% of the cells (data not shown). Strong binding to monocytes was also shown by Leong et al. [32] with the anti-MUC1 antibody E29.

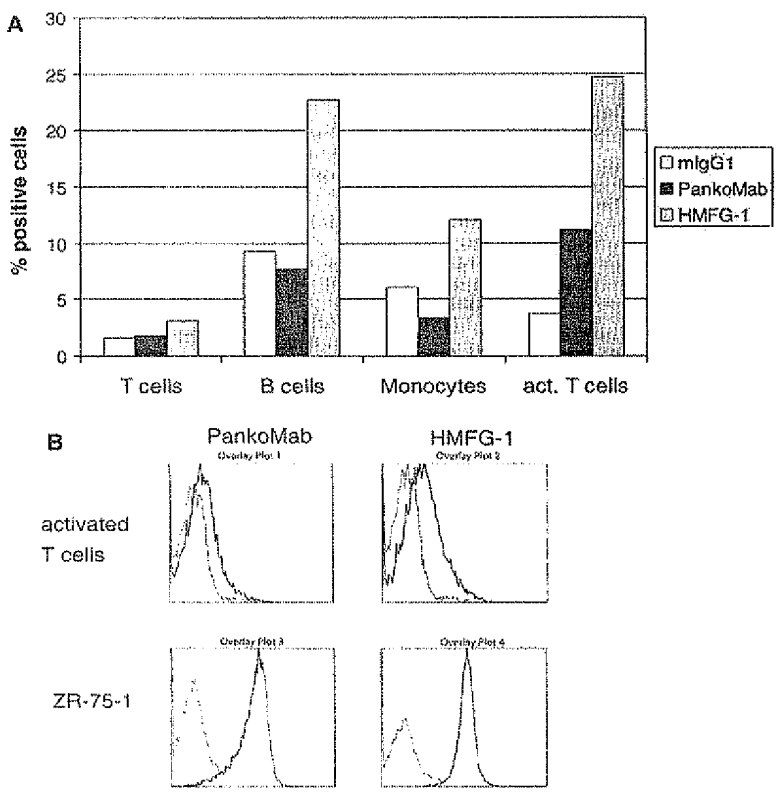

Fig. 2 Flow cytometry analysis of human PBMC. **a** PankoMab binding to MUC1 expressed on subsets of human PBMC was measured and compared to HMFG-1 and to an irrelevant murine IgG1 as a control. **b** Comparison of PankoMab and HMFG-1 binding to mitogen-activated human T cells and to the MUC1-expressing breast cancer cell line ZR-75-1

Detection of MUC1 in the sera of cancer patients

PankoMab was investigated for its potency to detect MUC1 in the sera of healthy persons and cancer patients by a sandwich ELISA using PankoMab for catching and detection. This was compared to HMFG-1 in an analogous setting and to conditions used in the commercially available CA15-3 assay (115D8 for catching and DF3 for detection). Table 2 summarises the data obtained. PankoMab did not bind above the threshold of 23 U/mL (level of healthy controls) to MUC1 in the sera from the pancreas of cancer patients and only in 33% of the colon carcinoma patients. In contrast, HMFG-1 bound to MUC1 in colorectal cancers detecting MUC1 above this threshold in 75% of the cases and the CA15-3 test detected even 83% of the cases. In addition, HMFG-1 recognised MUC1 in 38% of the sera of pancreatic cancer patients, while CA15-3 did not. MUC1 in normal sera was generally found to be below the threshold with all antibodies.

Internalisation and toxin-mediated cell death

Internalisation of PankoMab

Internalisation of PankoMab was analysed using ^{111}In-radiolabelled antibody. Experiments were performed after the saturation of the cell surface receptors with the antibody following the removal of the unbound antibody by a washing step. Figure 3a shows the specific saturable binding of PankoMab to T-47D cells at 4°C. The cell surface bound antibody was rapidly internalised at 37°C. Maxima of 80 and 60% were already reached after 1 h for the investigated cell lines T-47D and ZR-75-1, respectively, whereas less than 10% were taken up at 4°C over a period of at least 4 h. This is in accordance to published data [33]. Figure 3b shows as an example the time dependent internalisation of PankoMab by T-47D cells.

Toxin-mediated cell death by PankoMab

ToxiMab is a covalent conjugate of PankoMab and β-amanitin, which induces cell death after its internalisation by inhibition of RNA polymerase [5, 42]. Figure 4a shows the potent and specific T-47D tumour cell killing using ToxiMab with 0.25 μM of the toxin after 24 h incubation. For controls, neither the toxin alone nor a control IgG1 antibody coupled to β-amanitin, both used at the same toxin concentrations as ToxiMab, nor the antibody alone showed a killing effect to the cells (Fig. 4a). At least two individual experiments were performed. Induction of cell death is very rapid and already detectable after few minutes, and 70% cell death is measured already after 1 h as shown in the time curve in Fig. 4b. A concentration-dependent toxicity was measured for both the ToxiMab as well as the free toxin, whereby ToxiMab showed a ~30fold lower IC50 (toxin concentration necessary to kill 50% cells) at IC50 ~15 nM compared to the unconjugated toxin (IC50 ~500 nM). A concentration of down to 10 nM was already sufficient for cell killing within 24 h. Preincubation of the cells with a tenfold amount of PankoMab significantly reduced this effect, e.g. to about 50% after 4 h. Comparable results of the internalisation rate and cell killing were measured for another breast cancer cell line ZR-75-1 (data not shown).

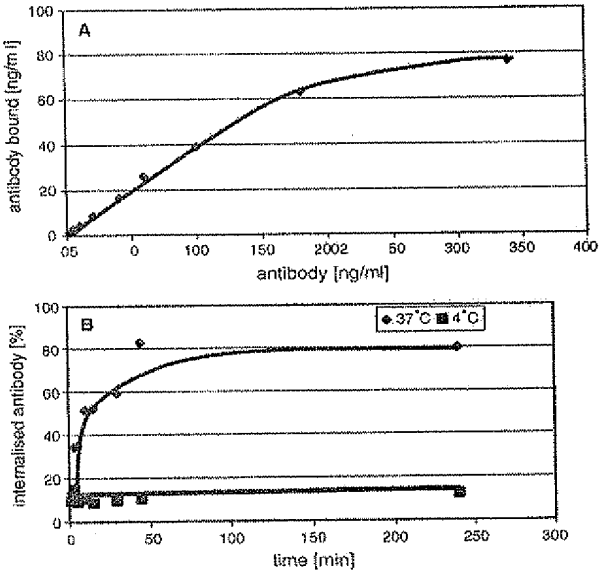

Fig. 3 Internalisation of PankoMab. **a** Saturation determination of PankoMab binding to T-47D cells at 4°C. **b** Time-dependent internalisation of ^{111}In-radiolabelled PankoMab by T-47D cells

ADCC activity of PankoMab

PankoMab specifically triggers ADCC of human PBMC against MUC1-positive tumour cell lines. Figure 5 shows

Table 2 Detection of serum MUC1 by a sandwich ELISA in the sera of cancer patients (cut-off level: 23 U/mL)

Catching antibody	Biotinylated detection antibody	Colon carcinoma		Pancreas carcinoma	
		Positive sera[a]	(%)	Positive sera[a]	(%)
115D8	DF3	20/24	83	0/16	0
PankoMab	PankoMab	8/24	33	0/16	0
HMFG-1	HMFG-1	18/23	78	6/16	38

[a]Number of positive sera/total number of sera tested

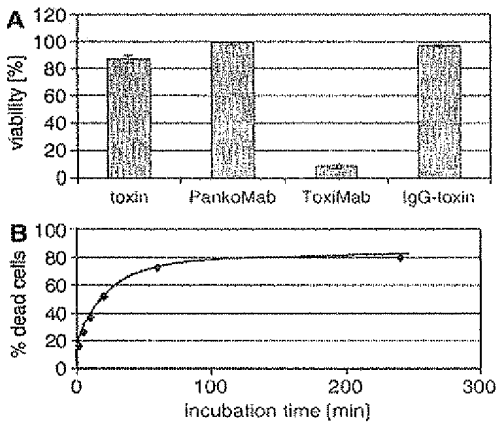

Fig. 4 Toxin-mediated cell death by PankoMab. a T-47D tumour cell killing using ToxiMab with 0.25 µM of the toxin after 24 h incubation. Toxin alone and a control IgG1 antibody coupled to β-amanitin were used at the same toxin concentrations as ToxiMab. PankoMab alone did not show a killing effect to the cells. Mean values ± SD are shown. b Kinetics of ToxiMab-induced death of T-47D cells

the concentration-dependent cell lysis of ZR-75-1 cells by PankoMab compared to HMFG-1 and a murine IgG1 control. PankoMab showed specific anti-tumour ADCC activity. The effectiveness of murine PankoMab in ADCC is lower compared to a chimeric version of the antibody (manuscript in preparation). However, compared to HMFG-1 detecting the same antigen, PankoMab is very effective. The MUC1-positive tumour cell line T-47D was also efficiently killed by PankoMab, whereas a concentration-dependent specific cell lysis was not detected using the MUC1-negative cell line MT-3, which served as a control (data not shown).

Discussion

After the discovery and description of the novel carbohydrate-induced conformational tumour-epitope of MUC1 [29, 30], the next logical step was to develop and characterise an antibody, which was able to distinguish between the novel tumour epitope and the non-tumour MUC1 epitopes for the future aim of developing improved antibody-based immunotherapeutics. From the panel of anti-MUC1 antibodies examined by Karsten et al. [29], only two antibodies, VU-11E2 and A76-A/C7, showed a strong but not absolute glycosylation dependency (*binding ratio of Tn-glycosylated* 30-*mer* [*APPAHGVTSAPDT*[*GalNAcα*]*RPAPGSTAPPAHGVTSA*] *to non-glycosylated* 30-*mer* [*APPAHGVTSAPDTRPAPGSTAPPAHGVTSA*] *of* > 6) which was combined with a strong additive length effect (*binding ratio TR4a : TR1a* > 2). In contrast, PankoMab was designed and selected to show an absolute glycosylation effect with a very strong binding to the Tn-glycosylated 30-mer and no detectable binding to the non-glycosylated 30-mer. PankoMab shows, in addition, the highest observed additive length effect (ratio 29.5), i.e. at least twice as high as that measured with other antibodies. This effect may be described as an induced fit of the conformational epitope structure caused by structural influences through elongating of the antigen by multiple PDTRP–glycosylated tandem repeats, which is given with natural MUC1. We refer to the epitope seen by PankoMab as *T*umour-*A*ssociated *MUC1* (*TA-MUC1*).

In contrast, HMFG-1 and C595, two antibodies currently in clinical development, do not bind specifically to TA-MUC1. Both antibodies show no glycosylation dependency in their binding [29], which is in accordance with the immunogen used for their generation (MUC1 of non-tumour origin) [39]. In addition, C595 is the only antibody known, which is independent of both the length of the MUC1 molecule and its glycosylation.

The difference in the biochemical fine-specificity of the various anti-MUC1 antibodies is also reflected in considerable differences in histology and serology [12, 29, 39], confirming the fact that all MUC1 antibodies have unique specificity patterns even when they bind nominally to the same immunodominant region. PankoMab stains strongly colorectal, gastric, breast, kidney, liver, lung, prostate, pancreas and ovarian carcinomas, as well as multiple myelomas and is negative with many normal tissues, including gastrointestinal tissues (manuscript in preparation). Thereby, it stains the large majority of carcinoma cases in these indications and reveals largely reduced staining with most other normal epithelia when compared to other MUC1 antibodies. In the few cases where PankoMab stains normal tissue, this staining is strictly located to the apical surface of the epithelial cells where it is not accessible to systemically applied antibodies.

An important feature of PankoMab is the almost complete lack of binding to cells of the haematopoietic system. None of the tested peripheral blood cells were stained by PankoMab. Only when the cells were mitogen-activated, about 7% of the activated T cells were weakly bound by PankoMab, a fact which we do not consider to

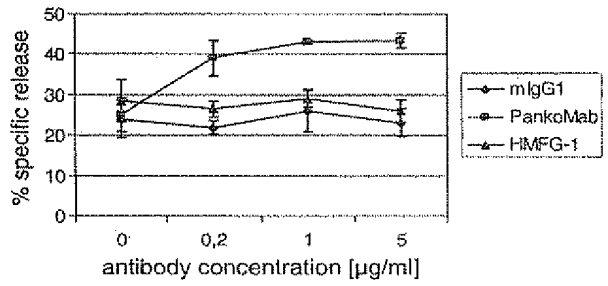

Fig. 5 ADCC activity of PankoMab. PankoMab-dependent killing of MUC1-positive tumour cell line ZR-75-1 using human PBMC as effector cells in a effector to target cell ratio of 80:1. HMFG-1 was not capable of mediating specific target cell killing compared to a murine IgG1 control antibody

be critical for immunotherapies. In contrast, HMFG-1 reacted with several types of blood cells, and to some of them in considerable proportions, which might become a problem in its systemic use in patients. Strong binding of HMFG-1 to stimulated T cells was also observed by Correa et al. [15]. This indicates that the glycosylation and the accessible epitopes of MUC1 on the tested blood cells are different from those of the novel tumour-epitope present on cancer cells. The non-identity between MUC1 expressed on haematopoietic cells and on tumour cells was confirmed by the results of other studies, which revealed complex binding patterns of the various MUC1 antibodies on haematopoietic cells, and confirmed the importance of subtle differences in the fine-specificity of anti-MUC1 antibodies [7, 15, 18].

Another important advantage of PankoMab is its very low binding to MUC1 shed from the tumour into the blood stream. Whereas the quantitative determination of shed MUC1 as a tumour marker is of great importance for monitoring of breast cancer patients in order to detect recurrence and to control the therapy of metastases [17, 22, 25, 44, 47], therapeutic antibodies should not be absorbed by shed MUC1. In this respect, PankoMab is much better than most other MUC1 antibodies. None of the 16 tested sera from patients with pancreatic carcinoma revealed binding to PankoMab above the threshold of 23 U/ml, while HMFG-1 binding surpassed the threshold level in 38% of the cases. In patients with colon cancer PankoMab detected MUC1 only in 33% of cases, whereas HMFG-1 led to significantly higher values in 78% of the patients. The detection level of MUC1 in normal sera was comparable for all antibodies tested (CA15-3 assay, HMFG-1 and PankoMab).

Low binding of PankoMab to normal cells and to shed MUC1 combines with high-affinity binding to tumour cells. Scatchard plot analyses at equilibrium revealed high affinity of PankoMab towards tumour cells ranging from $K_{ass} = 0.3–1 \times 10^9\ M^{-1}$ towards ZR75-1 and NM-D4 cells, respectively. The affinity to MUC1 on various tumour cells was about one magnitude higher than that of HMFG-1, which is considered to be one of the high-affinity MUC1 antibodies.

Another advantage of PankoMab is the very high number of about 10^6 antibody binding sites per tumour cell at saturation. In all the cases tested, PankoMab had at least 10 times more binding sites per cell than HMFG-1. The reason for this is not quite clear. HMFG-1 nominally binds to the same immunodominant region as PankoMab but is independent of glycosylation at PDTRP. In contrast, PankoMab has an increased tumour specificity due to its dependence on a tumour-specific, carbohydrate-induced conformation of the epitope. From this, one would expect that HMFG-1 would bind to at least the same number of binding sites than PankoMab. One possible explanation that PankoMab has more than 10 times more available binding sites on the tumour cell surface would be that the carbohydrate-induced conformation is modified by a further induced fit coming into effect with increasing length of the molecule, which is obviously the case with tumour MUC1 and is recognised by PankoMab, while the tumour glycosylation somehow decreases or at least does not increase the availability of the binding site for other antibodies like HMFG-1. This would be in accordance with the fact that the conformational epitope, a proposed knob structure [20], which is seen by HMFG-1 on multiple tandem repeats of naked peptides, is different from the carbohydrate-induced conformational epitope seen by PankoMab, which supposedly has a more rigid structure [29, 30, 41]. In any case, the results indicate that the carbohydrate-induced conformational epitope is not only the epitope with the higher tumour specificity, but also the one with the greater abundance on tumour cells, and hence an adequate target for an immunotherapy approach. This effect provides a further argument in favour of PankoMab. The affinities and numbers of binding sites of antibodies usually recommended for antibody immunotherapy, binding avidity in the nanomolar range and at least 10^5 antibody binding sites per tumour cell, are well exceeded by PankoMab.

Other important features for the suitability of a therapeutic antibody are their ability to internalise and/or to mediate potent ADCC activity. MUC1 is known to constitutively internalise and recycle [33]. However, internalisation rates of MUC1-bound antibodies vary widely for different antibodies, apparently depending on the epitope recognised [24, 36] and on MUC1 glycosylation [3]. Our results clearly show a very rapid and specific MUC1-mediated internalisation of PankoMab. About 20% of the cell surface-bound antibody molecules were internalised after 5 min of incubation at 37°C. The internalisation rate is comparable to that determined for MUC1 by a neuraminidase protection assay [33]. Internalisation is an essential precondition for the development of efficient therapeutic immunoconjugates with drugs, prodrugs and even radio-immunoconjugates [31, 37, 38]. The high potency of PankoMab for toxin-mediated cell killing was demonstrated by the effective and specific killing of MUC1-positive tumour cells using a PankoMab-toxin conjugate (Toximab).

Additionally, PankoMab revealed high and specific ADCC-mediated lytic activity using human PBMC as effector cells, whereas HMFG-1 was ineffective in the same concentration range. This is in accordance with Snijdewint et al. [43], where HMFG-1 was described to mediate a strong ADCC response as a humanised antibody, but was ineffective as a murine one. These results were obtained by comparing the antibodies in the murine IgG1 antibody format, which is known to mediate ADCC with human PBMC, albeit to a lower extent than chimeric or humanised antibodies with human constant regions. Other murine MUC1 antibodies such as DF3 were described not to mediate ADCC with human monocyte-derived macrophages [2]. PankoMab shows a much increased ADCC activity, as well as a very potent phagocytosis-enhancing activity when used as a chimeric human–mouse antibody (manuscript in preparation).

In conclusion, PankoMab is a novel anti-MUC1 antibody designed according to the latest knowledge of the nature of tumour MUC1. PankoMab, compared to other MUC1 antibodies and especially HMFG-1, has a higher specificity and affinity, detects more binding sites on tumour cells, reveals no binding to normal blood cells and is much less bound by shed MUC1. This set of largely improved characteristics together with the specific ADCC activity, its rapid internalisation and the potent specific killing of MUC1 positive tumour cells by toxin-coupled PankoMab renders it a promising new generation anti-MUC1 antibody for further clinical development as either naked, toxin-labelled or radiolabelled antibody for therapy and diagnosis of MUC1-positive tumours. Various chimeric and humanised formats of PankoMab are presently under investigation.

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